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13. ABSTRACT (Maximum 200 words)

Transforming growth factor ß (TGF-ß) is a potent growth inhibitor of normal breast epithelia. Evidence is accumulating that TGF-ß activity is critical to maintaining the negative paracrine and autocrine regulation of breast epithelial growth. Therefore, defects in the TGF-ß signaling pathways may result in uncontrolled growth of mammary epithelial cells and consequently contribute to carcinogenesis of the breast. TGF-ß acts through a direct mechanism as an inhibitor of proliferation in normal breast epithelium by inducing the expression of the cyclin/ckd inhibitors in several different cell types. Available evidence suggests that TGF-ß is also able to indirectly mediate its growth inhibitory effects on breast epithelia by inducing the secretion of insulin-like growth factor binding protein 3 (IGFBP-3). IGFBP's are a family of molecules which sequester and prevent insulin-like growth factors (IGF's) from binding and transducing mitogenic signals through the IGF receptors. Our goal is to dissect and elucidate the roles IGF, IGFBP-3, and TGF-ß in breast tumorigenesis. We are establishing if the induction of IGFBP-3 by TGF-ß in fibroblasts plays a significant role in regulating the growth of breast cancer epithelial cells. We are also investigating the molecular mechanism by which the expression of IGFBP-3 is regulated by TGF-ß in fibroblasts.

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2

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3

TABLE OF CONTENTS

INTRODUCTION

One of the most common cancers in women in the United States is breast cancer. In spite of some of the advances made in the field of breast cancer research over the past several years, the genes and pathways involved in the initiation, progression, and metastasis of breast cancer remain to be fully understood. Endocrine therapy continues to be the most common form of chemotherapy and has proven to be beneficial in approximately one-third of breast cancer patients. However, the drawback with these treatments is that they are only effective during the early stages of breast cancer. This is due to the fact that as breast tumors progress and accumulate more mutations, they eventually lose their ability to be regulated by hormones and other growth factors. In order to identify other possible therapeutic targets, it is necessary to further examine the development of breast cancer and identify other molecular mechanisms involved in the inhibition of tumor progression. The proposed study which examines the roles and interactions of the TGF-ß and IGF growth signals will significantly advance our understanding of the molecular abnormalities involved in breast cancers and will potentially lead to the development of novel therapeutic agents that slow or even halt cancer progression.

TGF-ß is a multifunctional peptide that plays a role in a wide variety of normal cellular functions including the regulation of proliferation, differentiation, extracellular matrix deposition, cell adhesion and migration (1). However, the effects of TGF-ß can be highly cell-type and even cell-state specific. In normal mammary epithelial cells, TGF-ß has been shown to be a potent inhibitor of growth through paracrine and autocrine pathways. TGF-ß acts through a direct mechanism as an inhibitor of proliferation by inducing the expression of the cyclin/cdk inhibitors such as p15, p21, and p27 in many cell types. Therefore, lesions to TGF-ß signaling pathways that disrupt the negative growth regulation of breast epithelia may contribute to mammary carcinogenesis and represent an obligatory step in neoplastic progression of breast epithelia. Indeed, loss of TGF-ß responsiveness of MCF7 breast cancer cells has been shown to correlate with a loss of expression in these cells of the TGF-ß signaling receptors (2). Loss of such autocrine control by TGF-ß represents an opportunity for malignant epithelia to increase proliferation in response to other positive growth factors, like IGF, and hormones, like estrogen.

Available evidence strongly suggests that an another aspect of TGF-ß-induced growth inhibition of breast cancer epithelia may involve countering the growth stimulatory effects of IGF by inducing the secretion of IGFBP-3. IGFBP's are a family of molecules which sequester and prevent IGF's from binding and transducing mitogenic signals through the IGF receptors. As interactions between breast epithelial and stromal cells have been implicated as being important in the genesis and proliferation of breast cancer, we wanted to test whether fibroblasts affect the growth of breast epithelia by secreting IGFBP-3 in response to TGF-ß. Such a phenomena, if perturbed, could contribute to mammary carcinogenesis.

Therefore, we have initiated a study aimed at determining the roles IGF, IGFBP-3, and TGF-ß have in breast tumorigenesis. In the past twelve months, our work has been focused on two main areas: analysis of the interactions between breast epithelial and fibroblast cells mediated by IGF, IGFBP-3 and TGF-ß and characterization of the molecular mechanism by which the expression of IGFBP-3 is regulated by TGF-ß. These studies were proposed as Technical Objectives 1 & 2 in the original proposal.

BODY

A. Development of Model Cell Culture System

As discussed above, TGF-ß may repress the growth of mammary epithelial cells through its induction of IGFBP-3 from stromal fibroblasts under normal and malignant conditions (3). This activity of TGF-ß may represent an indispensable mechanism by which the actions of growth stimulatory factors such as IGF are inhibited at the time when the cell cycle progression of epithelial cells is blocked by the TGF-ß signal. Furthermore, the effects of TGF-ß on the activity of IGF through its function of IGFBP-3 may represent an important aspect of interactions between mammary epithelial cells and stromal fibroblasts during normal mammary development as well as carcinogenesis. While several lines of evidence support this notion in the literature (4,5,6) we needed to test this hypothesis in a more direct way.

To study this, a cell culture system was established in which two MCF7 lines (PT3 and TEX sublines) were used as representative breast cancer cells. As negative control cells, the SKBR3 line was used since these cells lack responsiveness to IGF and TGF-ß. Based on the fact that TGF-ß induces IGFBP-3 in a variety of fibroblasts, we have been using MRC-9 fetal lung fibroblasts as a representative of stromal fibroblasts.   We found MRC-9 cells are not growth inhibited by TGF-ß, but we have shown that TGF-ß induces a 20-fold increase in both IGFBP-3 protein (Figure 1) and mRNA (even in the presence of cyclohexamide) (Figures 4,5).   We began with first confirming the response of our MCF7 cells to IGF-1, TGF-ß, and recombinant IGFBP-3. Following treatment with TGF-ß, our MCF7 cells were potently growth inhibited by 80% (as assayed by 3H-Thymidine incorporation). In contrast, IGF stimulated the growth of these cells by 30%. And, as expected, addition of equimolar amounts of recombinant IGFBP-3 was able to reverse the growth stimulatory effect of IGF1 completely.

Thus, we were ready to move on to our main experimental goal, which was to test if the conditioned media from MRC-9 cells containing IGFBP-3 induced by TGF-ß could function to block IGF mediated proliferation in MCF7 cells. To do this, we induced maximal production of IGFBP-3 in MRC-9 cells with the treatment of 50 pM TGF-ß1 for 48 hours and used the conditioned media to test its ability to inhibit IGF-induced proliferation in MCF7 cells.   Before addition to the MCF7 cells, the media was shown to indeed contain high levels of IGFBP-3. As shown in Figures 2 and 3, both MCF7 lines actually had a positive growth response to the control conditioned media, which was not unexpected since the MRC-9 cells were grown in complete growth media with 10% FBS and may also secrete growth factors the MCF7 cells respond to. Nevertheless, the increased growth response was reversed almost down to basal proliferation levels when the MCF7 cells were treated with the TGF-ß treated conditioned media from MRC-9 cells. Thus, it appeared that the conditioned media from the TGF-ß did have an anti-proliferative effect, which may be mediated by the secreted IGFBP-3.   Additionally, the TGF-ß treated conditioned MRC-9 media was able to significantly decrease the IGF1 induced growth response of the MCF7 cells.   As expected, the SKBR3 cells had no significant changes in proliferation with the conditioned medias.

Recommendations

Overall, it appears that the current protocol we have been using to test the effects of conditioned media from TGF-ß treated MRC-9 cells on MCF7 cells needs to be modified to reduce the background growth stimulatory effects of the media. With such background, it is difficult to interpret the effects, if any, secreted IGFBP-3 could be having on MCF7 proliferation. One way we are planning to modify our protocol is to change the media at the time of TGF-ß treatment of the MRC-9 cells to a serum-free or low serum condition, which is often done is these types of experiments. This way, growth factors carry-over to the MCF7 cells could be decreased.

In addition, to test whether the decreased proliferation on the MCF7 lines is due to the IGFBP-3 or the residual TGF-ß in the MRC-9 conditioned media, we are in the process of

6

repeating these experiments using neutralizing antibodies against TGF-ß and IGFBP-3. It is very possible that after 48 hours, the TGF-ß we added to the MRC-9 cells could still be having an growth inhibitory effect on the MCF7 cells. Preliminary results with a TGF-ß antibody, however, are inconsistent and suggest that there are many growth factors present in the conditioned media besides TGF-ß, which makes interpretation of this data difficult. However, if the growth inhibitory response is even partially due to IGFBP-3, use of a neutralizing antibody against IGFBP-3 in the same way may be able to block the observed growth inhibitory response. It is possible that the proliferation will increase even more with the addition of neutralizing antibodies. We are currently searching for such an antibody, as the one we use for Western blot analysis does not neutralize IGFBP-3 activity in the media. Nevertheless, further optimization of the system will need to be performed to surely conclude whether the secreted IGFBP-3 from the fibroblasts is adversely affecting the growth of breast cancer cells.

B. Promoter Analysis of IGFBP-3

As shown in Figure 2, we have found that TGF-ß potently induces IGFBP-3 transcript in MRC-9 cells. And, as Figure 3 shows, this induction of mRNA is not dependent on new protein synthesis (which is blocked by cyclohexamide), suggesting TGF-ß's effect on the IGFBP-3 gene is direct. To test the hypothesis that TGF-ß transcriptionally regulates the IGFBP-3 gene, we investigated whether the 5' proximal promoter region contained any TGF-ß-responsive elements. To this end, we obtained 2kb of the IGFBP-3 promoter upstream of the transcription start site, and cloned it into a reporter vector which drives the expression of a luciferase reporter gene (pGL2-Basic). Figure 5 diagrams the 5' deletions which were made of this promoter, in an effort to narrow down a TGF-ß responsive region. These constructs were transiently transfected into MRC-9 cells and TGF-ß induced luciferase activity was assayed. Unfortunately, after many attempts and conditions, no TGF-ß induced response was consistently observed for any of the IGFBP-3 promoter constructs (data not shown). In contrast, as a positive control, the common TGF-ß responsive reporter, p3TP-lux, gave a 5 fold induction of luciferase activity. Therefore, the TGF-ß responsive region was not located within -2kb of the promoter. Furthermore, sequences we obtained from another lab that corresponded to regions in introns 1 and 2 were not responsive.

To pursue this further, we performed a genomic library screen on a human placental library in the effort to obtain more sequence from introns 1-4, as many genes are known to contain enhancer elements in intronic sequences, as well as in sequences many kb away from their promoters. We screened the library using 800bp of IGFBP-3 coding sequence, and obtained genomic sequence that appeared to correlate with introns 1-3. We have had difficulty isolating and amplifying out the intronic sequences by PCR, and are still currently trying to clone these regions into the pGL2-Basic vector.

Considering that the regulation of IGFBP-3 mRNA by TGF-ß could possibly not involve transcription, we undertook a series of experiments to test whether TGF-ß regulated IGFBP-3 via mRNA stabilization. Actinomycin D is a potent inhibitor of transcription, and is used to assay for mRNA stability. Thus, MRC-9 cells were treated with or without TGF-ß in the presence of cyclohexamide for 0-10 hours. RNAse protection assays were performed, and the IGFBP-3 transcript was quantitated and normalized to the control GAPDH. Unfortunately, no real change in the 1/2 life of IGFBP-3 transcript was observed in the TGF-ß treated cells (data not shown). Thus, it appeared that transcription must be the mechanism of regulation of the IGFBP-3 gene by TGF-ß and more studies will need to be done to delineate the mechanism.

Recommendations

To definitively establish that the mechanism of IGFBP-3 mRNA accumulation is indeed achieved through an increased activity of the IGFBP-3 promoter in response to TGF-ß, we are planning to perform a nuclear run-off transcription assay. Although this can be a tricky assay, it is completely necessary before more studies on the promoter are pursued. If the transcription of

IGFBP-3 occurs in response to TGF-ß, then more genomic sequence will need to be obtained and analyzed. We are currently still working on the cloning the intronic sequences we did obtain. Furthermore, we still have the same human placental library, and a new screen can be undertaken to obtain more 5' promoter sequence beyond -2kb if necessary.
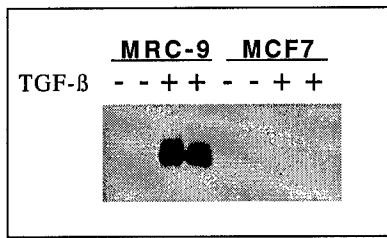
CONCLUSIONS

Cell growth is dictated by a delicate balance between positive and negative extracellular proliferative signals. Loss of this balance is a key factor leading to cancer. The goal of this research is to study the role of the antagonistic relationship between two opposing growth signals, IGF and TGFß, in mammary turmorigenesis. We set out to test a hypothesis that the induction of IGFBP-3 by TGF-ß in stromal fibroblasts is a mechanism by which TGF-ß regulates the growth of breast epithelial cells. To do this, first, a model cell culture system was established in which the effects of TGF-ß through IGFBP-3 are being studied. Our results suggest that recombinant IGFBP-3 is indeed able to block IGF-induced growth of breast cancer cells, however the effects of secreted IGFBP-3 from fibroblast media are still unclear. Growth inhibition does occur, but clarification of IGFBP-3's role in this effect still needs to be obtained. Additionally, we set out to define the molecular mechanisms by which TGF-ß induces IGFBP-3 by analyzing the promoter for TGF-ß regulatory elements. Unfortunately, no insight has been gained yet except that the regulation does not appear to occur through mRNA stability. Therefore, we hope to show that transcriptional regulation does occur and identify the regulatory elements used by TGF-ß.
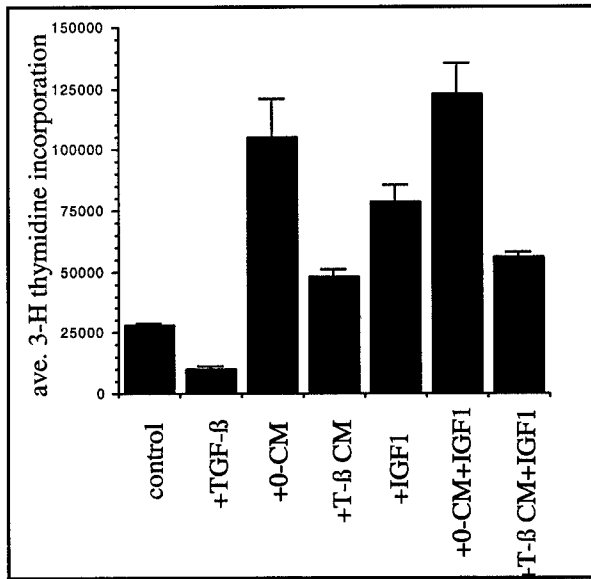
9

REFERENCES

1. Massague J 1990 The transforming growth factor-ß-family. Annu. Rev. Cell Biol. 6: 597-641.
2. Sun L, Wu G, Willson JKV, Zborowska E, Yang J, Rajkarunanayake I, Wang J, Gentry LE, Wang XF, Brattain MG 1994 Expression of transforming grwoth factor ß type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells. J. Biol. Chem. 269: 26449-26455.
3. Cullen KJ, Lippman ME 1992 Stromal-epithelial interactions in breast cancer. Cancer Treat. Res. 61: 413-431.
4. Conover CA, Clarkson JT, Durham SK, Bale LK 1991 Cellular actions of insulin-like growth factor binding protein-3. Current Directions in Insulin-like growth factor Research. CRC Press: 255-266.
5. Martin JL, Baxter RC 1991 Transforming growth factor-ß stimulates production of insulin-like growth factor-binding protein-3 by human skin fibroblasts. Endocrinology 128: 1425-1433.
6. Baxter RC, Martin JL 1991 Regulation and actions of insulin-like growth factor binding protein-3. Adv. Exp. Med. Biol. 293: 125-135.
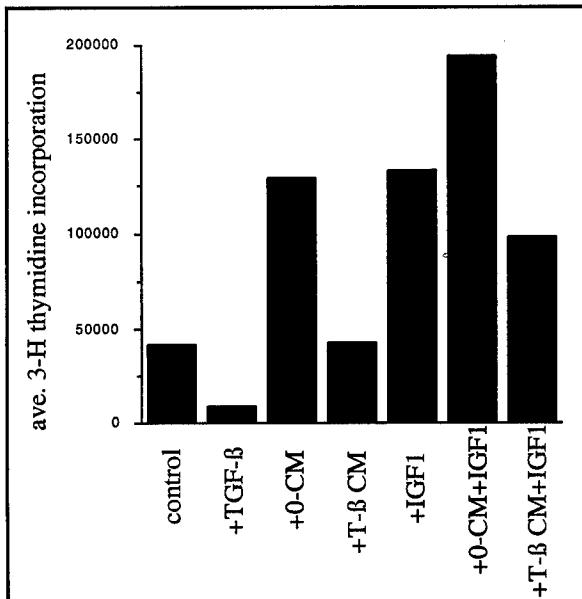
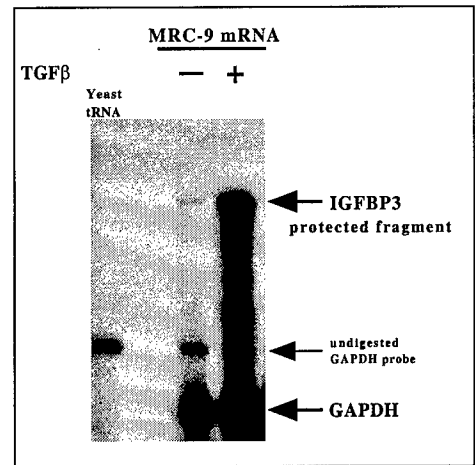**Figure 1**: Western blot analysis of IGFBP3 secreted protein induction in conditioned media fromMRC9 and MCF7 (a breast cancer epithelial cells) following 48 hours of TGFβ treatment.
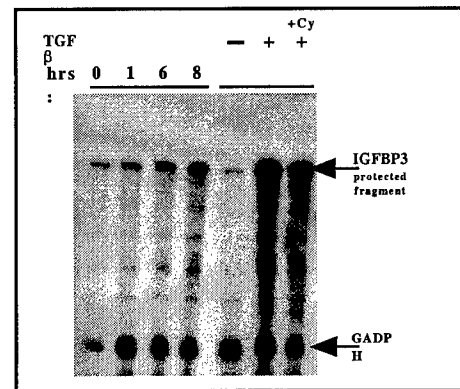

Figure 2: MCF-7/PT3 cell culture paracrine test of cell growth after treatment with TGF-ß, IGF1, or conditioned media (CM) from MRC-9 fibroblasts treated with or without TGF-ß.
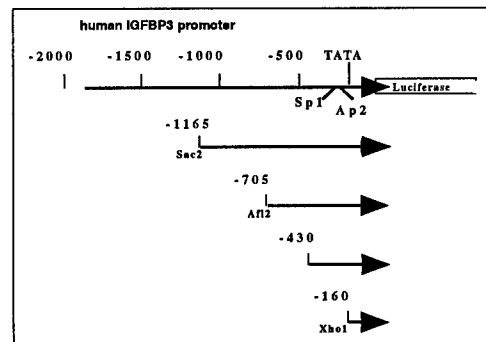

Figure 3: MCF-7/TEX cell culture paracrine test of cell growth after treatment with TGF-ß, IGF1, or conditioned media (CM) from MRC-9 fibroblasts treated with or without TGF-ß.

Figure 4: RNase protection analysis of IGFBP3 mRNA induction in MRC9 fibroblasts following 26 hours of TGFβ treatment.

Figure 5: RNase protection analysis of IGFBP3 mRNA induction in MRC9 cells following 0,1,6, and 8 hours of TGFβ treatment and after 8 hours of TGFβ addition in the presence of cycloheximide.

Figure 6: Diagram of the IGFBP3 promoter fragment obtained from Dr. D. Powell (-1800 bps) and the deletion constructs created from the Powell promoter. All promoter constructs were subcloned in front of the luciferase reporter gene.

11